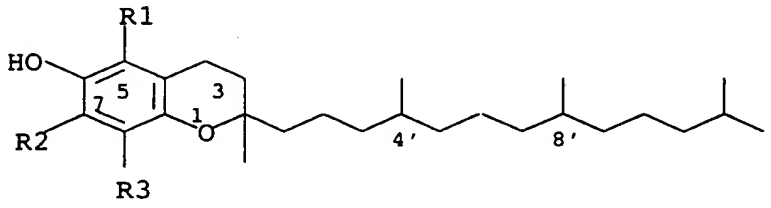
DNA sequence coding for a 1-deoxy-D-xylulose-5-phosphate synthase and overproduction thereof in plants

The present invention relates to the use of DNA sequences coding for a 1-deoxy-D-xylulose-5-phosphate synthase (DOXS) for producing plants with increased tocopherol, vitamin K, chlorophyll and/or carotenoid contents, specifically to the use of a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 or of a DNA sequence hybridizing with the latter, to the use of a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and of a DNA sequence SEQ ID No. 5 or DNA sequences hybridizing with the latter and coding for a 1-deoxy-D-xylulose-5-phosphate synthase (DOXS) and a p-dydroxyphenylpyruvate dioxygenase (HPPD) for producing plants with increased content of tocopherols, vitamin K, chlorophylls and/or carotenoids, to the use of a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and of a DNA sequence SEQ ID No. 7 or DNA sequences hybridizing with the latter and coding for a 1-deoxy-D-xylulose-5-phosphate synthase (DOXS) and a geranylgeranyl-pyrophosphate oxidoreductase (GGPPOR) for producing plants with increased content of tocopherols, vitamin K, chlorophylls and/or carotenoids, to the use of a DNA sequence SEQ ID No. 1 or SEQ ID No. 3, of a DNA sequence SEQ ID No. 5 and of a DNA sequence SEQ ID No. 7 or DNA sequences hybridizing with the latter and coding for a 1-deoxy-D-xylulose-5-phosphate synthase (DOXS), a hydroxyphenylpyruvate dioxygenase (HPPD) and a geranylgeranyl-pyrophosphate oxidoreductase (GGPPOR) for producing plants with increased content of tocopherols, vitamin K, chlorophylls and/or carotenoids, to processes for producing plants with increased tocopherol, vitamin K, chlorophyll and/or carotenoid contents, comprising a DNA sequence SEQ-ID No. 1 or SEQ ID No. 3; SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 5; SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 7 or a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 5 and SEQ ID No. 7, to the plants themselves produced in this way, and to the use of SEQ ID No. 1 or SEQ ID No. 3 for producing a test system for identifying DOXS inhibitors.

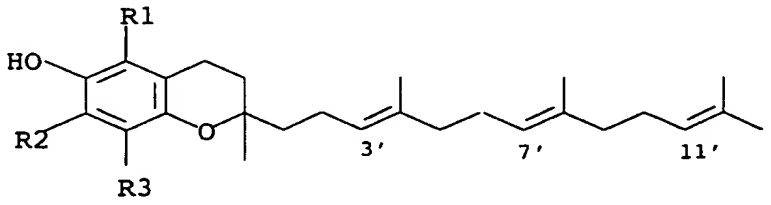
An important aim of molecular genetic work on plants to date has been the generation of plants with increased content of sugars, enzymes and amino acids. However, there is also commercial interest in the development of plants with increased content of vitamins, such as increasing the tocopherol content.

The eight compounds with vitamin E activity which occur in nature are derivatives of 6-chromanol (Ullmann's Encyclopedia of Industrial Chemistry, Vol. A 27 (1996), VCH Verlagsgesellschaft,

Chapter 4, 478–488, Vitamin E). The first group (1a-d) is derived from tocopherol, while the second group consists of derivatives of tocotrienol (2a- d):



1a, α–Tocopherol: $R^1 = R^2 = R^3 = CH_3$
1b, β–Tocopherol [148–03–8]: $R^1 = R^3 = CH_3$, $R^2 = H$
1c, γ–Tocopherol [54–28–4]: $R^1 = H$, $R^2 = R^3 = CH_3$
1d, δ–Tocopherol [119–13–1]: $R^1 = R^2 = H$, $R^3 = CH_3$



2a, α–Tocotrienol [1721–51–3]: $R^1 = R^2 = R^3 = CH_3$
2b, β–Tocotrienol [490–23–3]: $R^1 = R^3 = CH_3$, $R^2 = H$
2c, γ–Tocotrienol [14101–61–2]: $R^1 = H$, $R^2 = R^3 = CH_3$
2d, δ–Tocotrienol [25612–59–3]: $R^1 = R^2 = H$, $R^3 = CH_3$

The compound of great commercial importance is α–tocopherol.

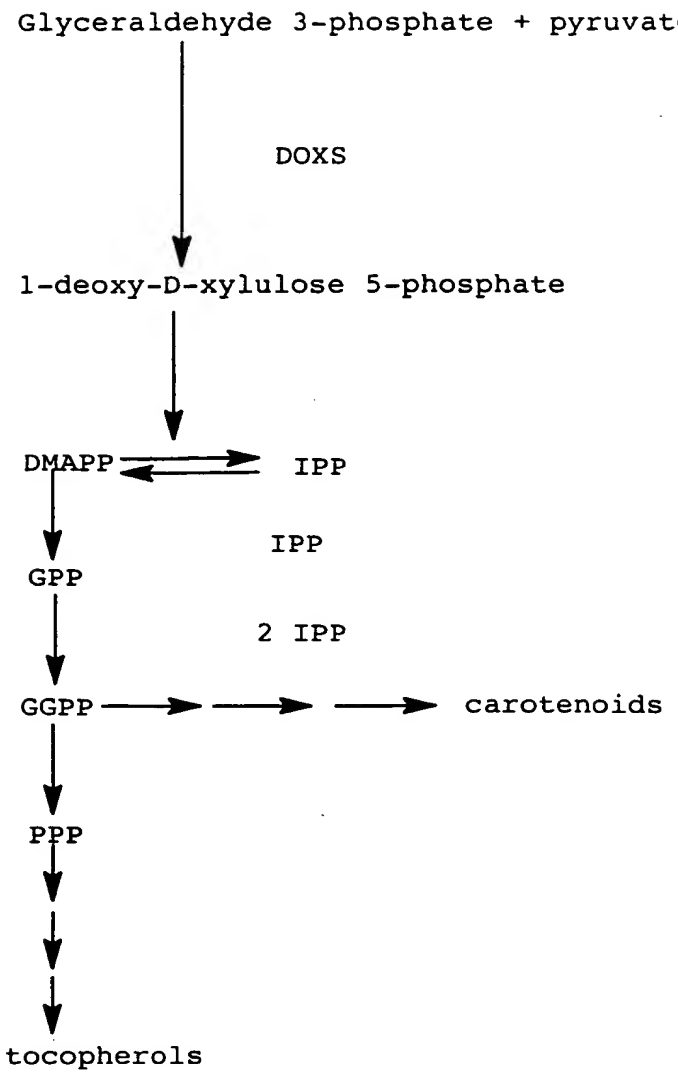
There are limits on the development of crop plants with increased tocopherol content through tissue culture or seed mutagenesis and natural selection. Thus, on the one hand, the tocopherol content must be measurable even in the tissue culture and, on the other hand, the only plants which can be manipulated by tissue culture techniques are those which can be regenerated to whole plants from cell cultures. In addition, crop plants may, after mutagenesis and selection, show unwanted properties which must be eliminated again by backcrossings, several times in some instances. Moreover increasing the tocopherol content by crossing would be retricted to plants of the same species.

For these reasons, the genetic engineering procedure of isolating, and transferring into target crop plants, an essential biosynthesis gene coding for tocopherol synthesis activity is

superior to the classical breeding method. The preconditions for this process are that the biosynthesis and its regulation are known and that genes which influence the biosynthetic activity are identified.

Isoprenoids or terpenoids consist of various classes of lipid-soluble molecules and are formed partly or completely of C_5-isoprene units. Pure prenyl lipids (e.g. carotenoids) consist of C skeletons derived exclusively from isoprene units, whereas mixed prenyl lipids (e.g. chlorophyll) have an isoprenoid side chain connected to an aromatic nucleus.

The biosynthesis of prenyl lipids starts from 3 x acetyl-CoA units, which are converted via ß-hydroxymethylglutaryl-CoA (HMG-CoA) and mevalonate into the initial isoprene unit (C_5), isopentenyl pyrophosphate (IPP). It has recently been shown by in vivo feeding experiments with C^{13} that a mevalonate-independent pathway is followed in various eubacteria, green algae and plant chloroplasts to produce IPP:



This entails hydroxyethylthiamine, which is produced by decarboxylation of pyruvate, and glyceraldehyde 3-phosphate (3-GAP) being converted, in a "transketolase" reaction mediated by 1-deoxy-D-xylulose-5-phosphate synthase, initially into 1-deoxy-D-xylulose-5-phosphate (Schwender et al., FEBS Lett. 414(1),129-134(1997); Arigoni et al., Proc.Natl.Acad.Sci USA 94(2), 10600-10605 (1997); Lange et al., Proc.Natl.Acad.Sci.USA 95(5), 2100-2104(1998); Lichtenthaler et al., FEBS Lett. 400(3), 271-274(1997). The latter is then converted by an intramolecular rearrangement into IPP (Arigoni et al., 1997). Biochemical data indicate that the mevalonate pathway operates in the cytosol and leads to the production of phytosterols. The antibiotic mevinolin, a specific inhibitor of mevalonate production, leads only to inhibition of sterol biosynthesis in the cytoplasm, whereas prenyl lipid production in the plastids is unaffected (Bach and Lichtenthaler, Physiol. Plant 59(1983), 50-60. By

contrast, the mevalonate-independent pathway has a plastidic localization and leads mainly to the production of carotenoids and plastidic prenyl lipids (Schwender et al., 1997; Arigoni et al, 1997).

IPP is in equilibrium with its isomer, dimethylallyl pyrophosphate (DMAPP). Condensation of IPP with DMAPP in a head-tail addition affords the monoterpene (C_{10}) geranyl pyrophosphate (GPP). Addition of further IPP units results in the sesquiterpene (C_{15}) farnesy pyrophosphate (FPP) and the diterpene (C_{20}) geranyl-geranyl pyrophosphate (GGPP). Linkage of two GGPP molecules results in the production of the C_{40} precursors for carotenoids. GGPP is transformed by a prenyl chain hydrogenase into phytyl pyrophosphate (PPP), the starting material for further production of tocopherols.

The ring structures of the mixed prenyl lipids which lead to the production of vitamins E and K comprise quinones whose initial metabolites are derived from the shikimate pathway. The aromatic amino acids phenylalanine and tyrosine are converted into hydroxyphenylpyruvate, which is transformed by dioxygenation into homogentisic acid. The latter is bound to PPP in order to produce 2-methyl-6-phytylquinol, the precursor of α-tocopherol and α-tocoquinone. Methylation steps with S-adenosylmethionine as methyl group donor result initially in 2,3-dimethyl-6-phytyl-quinol and then, by cyclization, in γ-tocopherol and, by methylation again, in α-tocopherol (Richter, Biochemie der Pflanzen, Georg Thieme Verlag Stuttgart, 1996).

Examples are to be found in the literature showing that manipulation of an enzyme can influence the direction of the metabolyte flux. It was possible in experiments with modified expression of phytoene synthase, which links two GGPP molecules together to give 15-cis-phytoene, to measure a direct effect on the amounts of carotenoids in these transgenic tomato plants (Fray and Grierson, Plant Mol.Biol.22(4),589-602(1993); Fray et al., Plant J., 8, 693-701(1995). As expected, transgenic tobacco plants with reduced amounts of phenylalanine-ammonium lyase show reduced phenylpropanoid amounts. The enzyme phenylalanine-ammonium lyase catalyzes the breakdown of phenylalanine and thus removes it from phenylpropanoid biosynthesis (Bate et al.,Proc. Natl. Acad. Sci USA 91 (16): 7608-7612 (1994); Howles et al., Plant Physiol. 112. 1617-1624(1996).

To date, little has been disclosed about increasing the metabolite flux in order to increase the tocopherol content of plants through overexpression of individual biosynthesis genes. There is merely a description in WO 97/27285 of modification of the tocopherol content by increased expression or by down-regulation of the enzyme p-hydroxyphenylpyruvate dioxygenase (HPPD).

It is an object of the present invention to develop a transgenic plant with increased content of tocopherols, vitamin K, chlorophylls and carotenoids.

We have found that this object is achieved by overexpression of a 1-deoxy-D-xylulose-5-phosphate sythase (DOXS) gene in the plants.

In order to increase the metabolite flux from primary metabolism into isoprenoid metabolism, the production of IPP as general starting substrate for all plastidic isoprenoids was increased. For this purpose, the DOXS activity in plants was increased by overexpression of the homologous gene (gene from organism of the same species). This can also be achieved by expressing a heterologous gene (gene from remote organisms). Nucleotide sequences from Arabidopsis thaliana DOXS (Acc. No. U 27099), rice (Acc. No. AF024512) and peppermint (Acc. No. AF019383) are described.

In one example 1 there is enhanced expression of the DOXS gene from Arabidopsis thaliana (SEQ ID No.:1; Mandel et al., Plant J. 9, 649-658(1996); Acc. No. U27099) in transgenic plants. Plastidic localization is ensured by the transit signal sequence present in the gene sequence. A suitable expression cassette is also a DNA sequence which codes for a DOXS gene which hybridizes with SEQ ID No. 1 and which is derived from other organisms such as, for example, E. coli (SEQ ID No.3) or, preferably, from other plants.

The GGPP which is now available in increased quantities is converted further in the direction of tocopherols and carotenoids.

Efficient production of carotenoids is essential for photosynthesis, where they serve together with chlorophylls as "light-collecting complexes" for better utilization of the energy of photons (Heldt, Pflanzenbiochemie. Spektrum Akademischer Verlag Heidelberg Berlin Oxford, 1996). In addition, carotenoids carry out important functions protecting from oxygen free radicals such as singlet oxygen, which they are able to return to

the ground state (Asada, 1994; Demming-Adams and Adams, Trends in Plant Sciences 1; 21-26(1996). A 1-deoxy-D-xylulose-5-phosphate synthase-defective Arabidopsis thaliana mutant showing an "albino phenotype" has been isolated (Mandel et al., 1996). It can be inferred from this that a reduced amount of carotenoids in the plastids has adverse effects on the plant.

We have found that the object is also achieved by overexpression of a 1-deoxy-D-xylulose-5-phosphate synthase (DOXS) gene and of a p-hydroxyphenylpyruvate dioxygenase (HPPD) gene in the plants, see Figure 1.

In order to increase the metabolite flux from primary metabolism into isoprenoid metabolism, the production of IPP as general starting substrate for all plastidic isoprenoids was increased. For this purpose, the DOXS activity in transgenic tobacco and oilseed rape plants was increased by overexpression of the DOXS from E. coli. This can be achieved by expression of homologous or other heterologous genes.

The D-1-deoxy-xylulose 5-phosphate which is now available in increased quantities is converted further in the direction of tocopherols and carotenoids.

In addition, the production of homogentisic acid further intensifies the metabolite flux in the direction of phytylquinones and thus tocopherol, see Figure 1. Homogentisic acid is produced from p-hydroxyphenylpyruvate by the enzyme p-hydroxyphenylpyruvate dioxygenase (HPPD). cDNAs coding for this enzyme have been described from various organisms such as, for example, from microorganisms, from plants and from humans.

In Example 11 there was for the first time overexpression of the HPPD gene from Streptomyces avermitilis (Denoya et al., J. Bacteriol. 176(1994), 5312-5319; SEQ ID No. 5) together with the DOXS from E. coli SEQ ID No. 3 in plants and plant plastids.

The increase in the plastidic IPP production leads to enhanced production of all plastidic isoprenoids. The increased provision of homogentisic acid ensures that sufficient substrate is available for the production of tocopherols in the plastids. This homogentisate which is now available in increased quantities can in turn be converted in the transgenic plants with the amount, which is increased due to the overexpression of DOXS, of phytyl diphosphate (PPP). PPP occupies a key position, in this connection, because it serves on the one hand as starting

substrate for chlorophylls and phylloquinones, and on the other hand for tocopherols.

The transgenic plants are produced by transformation of the plants with a construct containing the DOXS and HPPD genes. Tobacco and oilseed rape were employed as model plants for the production of tocopherols, vitamin K, chlorophylls and carotenoids.

The invention also relates to the use of the DNA sequences SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 5, which code for a DOXS or HPPD or functional equivalents thereof, for producing a plant with increased tocopherol, vitamin K, chlorophyll and/or carotenoid contents. The nucleic acid sequences may in these cases be, for example, DNA or cDNA sequences. Coding sequences suitable for insertion into an expression cassette are, for example, those coding for a DOXS or HPPD and conferring on the host the ability to overproduce tocopherol.

The expression cassettes additionally comprise regulatory nucleic acid sequences which control the expression of the coding sequence in the host cell. In a preferred embodiment, an expression cassette comprises upstream, i.e. at the 5' end of the coding sequence, a promoter and downstream, i.e. at the 3' end, a polyadenylation signal and, where appropriate, further regulatory elements which are operatively linked to the coding sequence for the DOXS or HPPD gene located in between.

An expression cassette is produced by fusing a suitable promoter to a suitable DOXS or HPPD DNA sequence and preferably a DNA which is inserted between promoter and DOXS or HPPD DNA sequence and codes for a chloroplast-specific transit peptide, and a polyadenylation signal by conventional recombination and cloning techniques as described, for example, in T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989), and in T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987).

It is also possible to use expression cassettes whose DNA sequence codes for a DOXS or HPPD fusion protein, where part of the fusion protein is a transit peptide which controls translocation of the polypeptide. Transit peptides which are specific for chloroplasts and which are eliminated enzymatically

from the DOXS or HPPD part after translocation of the DOXS or HPPD gene into the chloroplasts are preferred. The particularly preferred transit peptide is derived from the plastidic transketolase (TK) or a functional equivalent of this transit peptide (for example the transit peptide of the small subunit of rubisco or ferredoxin-NADP oxidoreductase).

The fused expression cassette coding for a DOXS gene and an HPPD gene is preferably cloned into a vector, for example pBin19, which is suitable for transformation of Agrobacterium tumefaciens.

The invention further relates to the use of an expression cassette comprising DNA sequences SEQ ID No. 1 or SEQ-ID No. 3 and SEQ ID No. 5 or DNA sequences hybridizing with the latter for the transformation of plants or cells, tissues or parts of plants. The preferred aim of the use is to increase the tocopherol, vitamin K, chlorophyll and carotenoid contents of the plant.

It is moreover possible, depending on the choice of the promoter, for expression to take place specifically in the leaves, in the seeds or other parts of the plant. The present invention further relates to such transgenic plants, propagation material thereof and cells, tissues or parts of these plants.

The invention additionally relates to transgenic plants transformed with an expression cassette comprising the sequence SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 5 or DNA sequences hybridizing with the latter, and transgenic cells, tissues, parts and propagation material of such plants. Particular preference is given in this connection to transgenic crop plants such as, for example, barley, wheat, rye, corn, oats, soybean, rice, cotton, sugarbeet, canola, sunflower, flax, hemp, potato, tobacco, tomato, oilseed rape, alfalfa, lettuce and the various tree, nut and vine species.

The invention further relates to:

- Process for transforming a plant, which comprises introducing expression cassettes comprising a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and a DNA sequence SEQ ID No. 5 or DNA sequences hybridizing with the latter into a plant cell, into callus tissue, a whole plant or protoplasts of plants,

- Use of the DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 5 or DNA sequences hybridizing with the latter to produce plants with increased tocopherol, vitamin K, chlorophyll and/or carotenoid contents by expression of a DOXS and an HPPD DNA sequence in plants.

The object have also been achieved by overexpression of a 1-deoxy-D-xylulose-5-phosphate synthase (DOXS) gene and of a geranylgeranyl-pyrophosphate oxidoreductase (GGPPOR) gene in the plants, see Figure 1.

In order to increase the metabolite flux from primary metabolism into isoprenoid metabolism, the production of IPP as general starting substrate for all plastidic isoprenoids was increased. For this purpose, the DOXS activity in transgenic tobacco and oilseed rape plants was increased by overexpression of the DOXS from E. coli. This can be achieved by expression of homologous or other heterologous genes.

In order to convert the GGPP which is now available in increased quantities in the direction of tocopherols and carotenoids, in a further step essential to the invention in addition the activity of the enzyme geranylgeranyl-pyrophosphate oxidoreductase is increased by overexpression of a corresponding gene. This measure achieves an increased production of phytyl pyrophosphate through increased conversion of geranylgeranyl pyrophosphate into phytyl pyrophosphate.

This is done, for example, by enhanced expression of the GGPPOR gene from Arabidopsis thaliana (SEQ ID No. 7) in transgenic plants. In order to ensure plastidic localization, a transit signal sequence is put in front of the Arabidopsis GGPPOR. Also suitable as expression cassette is a DNA sequence coding for a GGPPOR gene which hybridizes with SEQ ID No. 7 and which is derived from other organisms or from other plants.

Example 15 describes the cloning of the GGPPOR gene from Arabidopsis thaliana.

Increasing the plastidic 1-deoxy-D-xylulose 5-phosphate and phytyl pyrophosphate production leads to increased production of all plastidic isoprenoids, so that sufficient substrate for the production of tocopherols, chlorophylls, vitamin K and phylloquinones is available in the plastids.

The transgenic plants are produced by transformation of the plants with a construct containing the DOXS and GGPPOR genes. Tobacco and oilseed rape were employed as model plants for the production of tocopherols, vitamin K, chlorophylls and carotenoids.

The invention also relates to the use of the DNA sequences SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 7, which code for a DOXS or GGPPOR or functional equivalents thereof, for producing plants with increased tocopherol, vitamin K, chlorophyll and/or carotenoid contents. The nucleic acid sequences may in these cases be, for example, DNA or cDNA sequences. Coding sequences suitable for insertion into an expression cassette are, for example, those coding for a DOXS or GGPPOR and conferring on the host the ability to overproduce tocopherol.

The expression cassettes additionally comprise regulatory nucleic acid sequences which control the expression of the coding sequence in the host cell. In a preferred embodiment, an expression cassette comprises upstream, i.e. at the 5' end of the coding sequence, a promoter and downstream, i.e. at the 3' end, a polyadenylation signal and, where appropriate, further regulatory elements which are operatively linked to the coding sequence for the DOXS or GGPPOR gene located in between. Operative linkage means sequential arrangement of promoter, coding sequence, terminator and, where appropriate, further regulatory elements in such a way that each of the regulatory elements can properly carry out its function in the expression of the coding sequence. The sequences which are preferred for the operative linkage, but are not restricted thereto, are targeting sequences to ensure subcellular localization in the apoplast, in the vacuole, in plastids, in the mitochondrion, in the endoplasmic reticulum (ER), in the cell nucleus, in elaioplasts or other compartments and translation enhancers such as the 5' leader sequence from tobacco mosaic virus (Gallie et al., Nucl. Acids Res. 15 (1987), 8693-8711).

For example, the plant expression cassette can be incorporated into the tobacco transformation vector pBinAR-Hyg. Fig. 1 shows the tobacco transformation vectors pBinAR–Hyg with the 35S promoter (A) and pBinAR–Hyg with the seed-specific promoter phaseolin 796 (B):

- HPT: hygromycin phosphotransferase
- OCS: octopine synthase terminator
- PNOS: nopaline synthase promoter

- also drawn in are those restriction cleavage sites which cut the vector only once.

Suitable promoters for the expression cassette are in principle all promoters able to control expression of foreign genes in plants. Preferably used is, in particular, a plant promoter or a promoter derived from a plant virus. The CaMV 35S promoter from cauliflower mosaic virus (Franck et al., Cell 21 (1980), 285-294) is particularly preferred. This promoter contains, as is known, different recognition sequences for transcriptional effectors which, in their totality, lead to permanent and constitutive expression of the inserted gene (Benfey et al., EMBO J. 8 (1989), 2195-2202).

The expression cassette may also contain a chemically inducible promoter by which expression of the exogenous DOXS or GGPPOR gene in the plant can be controlled at a particular time. Promoters of this type, such as the PRP1 promoter (Ward et al., Plant. Mol. Biol. 22 (1993), 361-366), a promoter inducible by salicylic acid (WO 95/19443), a benzenesulfonamide-inducible (EP–A 388186), a tetracycline-inducible (Gatz et al., (1992) Plant J. 2, 397-404), an abscisic acid-inducible (EP–A 335528) and an ethanol- or cyclohexanone-inducible (WO 93/21334) promoter, inter alia, can be used.

Further particularly preferred promoters are those which ensure expression in tissues or parts of plants in which the biosynthesis of tocopherol or its precursors takes place. Particular mention should be made of promoters which ensure leaf-specific expression. Mention should be made of the promoter of cytosolic FBPase from potato or the ST-LSI promoter from potato (Stockhaus et al., EMBO J. 8 (1989) 2445 - 245).

An expression cassette is produced by fusing a suitable promoter to a suitable DOXS or GGPPOR DNA sequence and, preferably, to a DNA which is inserted between promoter and DOXS or GGPPOR DNA sequence and which codes for a chloroplast-specific transit peptide, and to a polyadenylation signal, by conventional recombination and cloning techniques as described, for example, in T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and in T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987).

It is also possible to use expression cassettes whose DNA sequence codes for a DOXS or GGPPOR fusion protein, where part of the fusion protein is a transit peptide which controls translocation of the polypeptide. Transit peptides specific for chloroplasts are particularly preferred, and these are eliminated enzymatically from the DOXS or GGPPOR part after translocation of the DOXS or GGPPOR gene into the chloroplasts. The particularly preferred transit peptide is derived from the plastidic transketolase (TK) or a functional equivalent of this transit peptide (e.g. the transit peptide of the small subunit of rubisco or ferredoxin-NADP oxidoreductase).

The fused expression cassette coding for a DOXS gene or a GGPPOR gene is preferably cloned into a vector, for example pBin19, which is suitable for transforming Agrobacterium tumefaciens.

The invention further relates to the use of an expression cassette comprising DNA sequences SEQ ID No. 1 or SEQ-ID No. 3 and SEQ ID No. 7 or DNA sequences hybridizing with the latter for the transformation of plants or cells, tissues or parts of plants. The preferred aim of the use is to increase the tocopherol, vitamin K, chlorophyll and carotenoid contents of the plant.

It is moreover possible, depending on the choice of the promoter, for expression to take place specifically in the leaves, in the seeds or other parts of the plant. The present invention further relates to such transgenic plants, propagation material thereof and cells, tissues or parts of these plants.

The invention additionally relates to transgenic plants transformed with an expression cassette comprising the sequence SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 7 or DNA sequences hybridizing with the latter, and transgenic cells, tissues, parts and propagation material of such plants. Particular preference is given in this connection to transgenic crop plants such as, for example, barley, wheat, rye, corn, oats, soybean, rice, cotton, sugarbeet, canola, sunflower, flax, hemp, potato, tobacco, tomato, oilseed rape, alfalfa, lettuce and the various tree, nut and vine species.

The invention further relates to:

- Process for transforming a plant, which comprises introducing expression cassettes comprising a DNA sequence SEQ ID No. 1 or a DNA sequence SEQ ID No. 3 and a SEQ ID No. 7 or DNA

sequences hybridizing with the latter into a plant cell, into callus tissue, a whole plant or protoplasts of plants,

- Use of the DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 7 or DNA sequences hybridizing with the latter to produce plants with increased tocopherol, vitamin K, chlorophyll and/or carotenoid contents by expression of a DOXS and a GGPPOR DNA sequence in plants.

The object have also been achieved by overexpression of a 1-deoxy-D-xylulose-5-phosphate synthase (DOXS) gene, a p-hydroxyphenylpyruvate dioxygenase (HPPD) gene and a geranylgeranyl-pyrophosphate oxidoreductase (GGPPOR) gene in the plants, see Figure 1.

In order to increase the metabolite flux from primary metabolism into isoprenoid metabolism, the production of IPP as general starting substrate for all plastidic isoprenoids was increased. For this purpose, the DOXS activity was increased by overexpression of the DOXS from E. coli in transgenic tobacco and oilseed rape plants. This can also be achieved by expressing homologous or other heterologous DOXS genes - such as, for example, a DNA sequence SEQ ID No. 1.

The D-1-deoxy-xylulose 5-phosphate which is now available in increased quantities is converted further in the direction of geranylgeranyl pyrophosphate.

In order to convert the GGPP which is now available in increased quantities in the direction of tocopherols and carotenoids, in a further step essential to the invention in addition the activity of the enzyme geranylgeranyl pyrophosphate oxidoreductase is increased by overexpression of a corresponding homologous or heterologous gene. This measure achieves an increased production of phytyl pyrophosphate through increased conversion of geranylgeranyl pyrophosphate into phytyl pyrophosphate.

This done, for example, by enhanced expression of the GGPPOR gene from Arabidopsis thaliana (SEQ ID No. 7) in transgenic plants. In order to ensure plastidic localization, a transit signal sequence is put in front of the Arabidopsis GGPPOR. Also suitable as expression cassette is a DNA sequence coding for a GGPPOR gene which hybridizes with SEQ ID No. 7 and which is derived from other organisms or from other plants.

Example 15 describes the cloning of the GGPPOR gene from Arabidopsis thaliana.

In order to convert the PPP which is now available in increased quantities in the direction of tocopherol and carotenoids, in a further step essential to the invention in addition the activity of the enzyme p-hydroxylphenylpyruvate dioxygenase (HPPD) is increased by overexpression of a corresponding homologous or heterologous gene. This measure achieves increased production of homogentisic acid by increased conversion of hydroxyphenylpyruvate into homogentisic acid.

cDNAs coding for this enzyme have been described from various organisms such as, for example, from microorganisms, from plants and from humans.

Example 10 describes the cloning of the HPPD gene from Streptomyces avermitilis (Denoya et al., J. Bacteriol. 176(1994), 5312-5319; SEQ ID No. 5). In order to ensure a plastidic localization, a transit signal sequence is put in front of the Streptomyces HPPD. Also suitable as expression cassette is a DNA sequence which codes for an HPPD gene which hybridizes with SEQ ID No. 5 and is derived from other organisms or from plants.

The increase in the plastidic D-1-deoxy-xylulose 5-phosphate, the phytyl pyrophosphate and the homogentisic acid production leads to increased production of all plastidic isoprenoids. The increased provision of these precursors ensures that sufficient substrate is available for the production of tocopherols, chlorophylls, vitamin K and phylloquinones in the plastids.

The transgenic plants according to the invention are produced by transforming the plants with a construct containing the DOXS, the HPPD gene and the GGPPOR gene (Example 17). Tobacco and oilseed rape were employed as model plants for producing tocopherols, vitamin K, chlorophylls and carotenoids.

The invention relates to the use of the DNA sequences SEQ ID No. 1 or SEQ ID No. 3, SEQ ID No. 5 and SEQ-ID No. 7, which code for a DOXS, an HPPD and a GGPPOR or functional equivalents thereof to produce a plant with increased tocopherol, vitamin K, chlorophyll and/or carotenoid contents. The nucleic acid sequences may in these cases be, for example, DNA or cDNA sequences. Coding sequences suitable for insertion into an expression cassette are, for example, those coding for a DOXS, an HPPD and a GGPPOR and conferring on the host the ability to overproduce tocopherol.

The expression cassettes additionally comprise regulatory nucleic acid sequences which control the expression of the coding sequence in the host cell. In a preferred embodiment, an expression cassette comprises upstream, i.e. at the 5' end of the coding sequence, a promoter and downstream, i.e. at the 3' end, a polyadenylation signal and, where appropriate, further regulatory elements which are operatively linked to the coding sequence for the DOXS, the HPPD or the GGPPOR gene located in between. Operative linkage means sequential arrangement of promoter, coding sequence, terminator and, where appropriate, further regulatory elements in such a way that each of the regulatory elements can properly carry out its function in the expression of the coding sequence. The sequences which are preferred for the operative linkage, but are not restricted thereto, are targeting sequences to ensure subcellular localization in the apoplast, in the vacuole, in plastids, in the mitochondrion, in the endoplasmic reticulum (ER), in the cell nucleus, in elaioplasts or other compartments and translation enhancers such as the 5' leader sequence from tobacco mosaic virus (Gallie et al., Nucl. Acids Res. 15 (1987), 8693-8711).

For example, the plant expression cassette can be incorporated into the tobacco transformation vector pBinAR-Hyg. Fig. 2 shows the tobacco transformation vectors pBinAR—Hyg with the 35S promoter (A) and pBinAR—Hyg with the seed-specific promoter phaseolin 796 (B):

- HPT: hygromycin phosphotransferase
- OCS: octopine synthase terminator
- PNOS: nopaline synthase promoter
- also drawn in are those restriction cleavage sites which cut the vector only once.

Suitable promoters for the expression cassette are in principle all promoters able to control expression of foreign genes in plants. Preferably used is, in particular, a plant promoter or a promoter derived from a plant virus. The CaMV 35S promoter from cauliflower mosaic virus (Franck et al., Cell 21 (1980), 285-294) is particularly preferred. This promoter contains, as is known, different recognition sequences for transcriptional effectors which, in their totality, lead to permanent and constitutive expression of the inserted gene (Benfey et al., EMBO J. 8 (1989), 2195-2202).

The expression cassette may also contain a chemically inducible promoter by which expression of the exogenous DOXS, HPPD and GGPOR gene in the plant can be controlled at a particular time.

Promoters of this type, such as the PRP1 promoter (Ward et al., Plant. Mol. Biol. 22 (1993), 361-366), a promoter inducible by salicylic acid (WO 95/19443), a benzenesulfonamide-inducible (EP-A 388186), a tetracycline-inducible (Gatz et al., (1992) Plant J. 2, 397-404), an abscisic acid-inducible (EP-A 335528) and an ethanol- or cyclohexanone-inducible (WO 93/21334) promoter, inter alia, can be used.

Further particularly preferred promoters are those which ensure expression in tissues or parts of plants in which the biosynthesis of tocopherol or its precursors takes place. Particular mention should be made of promoters which ensure leaf-specific expression. Mention should be made of the promoter of cytosolic FBPase from potato or the ST-LSI promoter from potato (Stockhaus et al., EMBO J. 8 (1989) 2445 - 245).

An expression cassette is produced by fusing a suitable promoter to a suitable DOXS, HPPD and GGPPOR DNA sequence and, preferably, to a DNA which is inserted between promoter and DOXS, HPPD and GGPOR DNA sequence and which codes for a chloroplast-specific transit peptide, and to a polyadenylation signal, by conventional recombination and cloning techniques as described, for example, in T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and in T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987).

It is also possible to use expression cassettes whose DNA sequence codes for a DOXS, HPPD and GGPOR fusion protein, where part of the fusion protein is a transit peptide which controls translocation of the polypeptide. Transit peptides specific for chloroplasts are particularly preferred, and these are eliminated enzymatically from the DOXS, HPPD and GGPPOR part after translocation of the DOXS, HPPD and GGPOR gene into the chloroplasts. The particularly preferred transit peptide is derived from the plastidic transketolase (TK) or a functional equivalent of this transit peptide (e.g. the transit peptide of the small subunit of rubisco or ferredoxin-NADP oxidoreductase).

The fused expression cassette coding for a DOXS gene, an HPPD gene or a GGPPOR gene is preferably cloned into a vector, for example pBin19, which is suitable for transforming Agrobacterium tumefaciens.

The invention further relates to the use of an expression cassette comprising DNA sequences SEQ ID No. 1 or SEQ-ID No. 3, SEQ ID No. 5 and SEQ ID No. 7 or DNA sequences hybridizing with the latter for the transformation of plants or cells, tissues or parts of plants. The preferred aim of the use is to increase the tocopherol, vitamin K, chlorophyll and carotenoid contents of the plant.

It is moreover possible, depending on the choice of the promoter, for expression to take place specifically in the leaves, in the seeds or other parts of the plant. The present invention further relates to such transgenic plants, propagation material thereof and cells, tissues or parts of these plants.

The invention additionally relates to transgenic plants transformed with an expression cassette comprising the sequence SEQ ID No. 1 or SEQ ID No. 3, SEQ ID No. 5 and SEQ ID No. 7 or DNA sequences hybridizing with the latter, and transgenic cells, tissues, parts and propagation material of such plants. Particular preference is given in this connection to transgenic crop plants such as, for example, barley, wheat, rye, corn, oats, soybean, rice, cotton, sugarbeet, canola, sunflower, flax, hemp, potato, tobacco, tomato, oilseed rape, alfalfa, lettuce and the various tree, nut and vine species.

The invention further relates to:

- Processes for transforming a plant, which comprises introducing expression cassettes comprising a DNA sequence SEQ ID No. 1 or SEQ ID No. 3, a DNA sequence SEQ ID No. 5 and a DNA sequence SEQ ID No. 7 or DNA sequences hybridizing with the latter into a plant cell, into callus tissue, a whole plant or protoplasts of plants,

- Use of the DNA sequence SEQ ID No. 1 or SEQ ID No. 3, SEQ ID No. 5 and SEQ ID No. 7 or DNA sequences hybridizing with the latter to produce plants with increased tocopherol, vitamin K, chlorophyll and/or carotenoid contents by expression of a DOXS, an HPPD and an GGPPOR DNA sequence in plants.

It was therefore an additional object of the present invention to develop a test system for identifying DOXS inhibitors.

This object has been achieved by expressing a DOXS gene from Arabidopsis or E. coli, or DNA sequences hybridizing therewith, and subsequently testing chemicals for inhibition of the DOXS enzyme activity.

The transgenic plants are produced by transforming the plants with a construct containing the DOXS gene. Arabidopsis and oilseed rape were employed as model plants for the production of tocopherols, vitamin K, chlorophylls and carotenoids.

Cloning of the complete DOXS gene from Arabidopsis takes place by isolating the cDNA (SEQ ID No. 1) specific for the DOXS gene.

The invention relates to the use of the DNA sequence SEQ ID No. 1 or SEQ ID No. 3 which codes for a DOXS or functional equivalent thereof for producing a plant with increased tocopherol, vitamin K, chlorophyll and/or carotenoid content. The nucleic acid sequence can moreover be, for example, a DNA or cDNA sequence. Examples of coding sequences suitable for insertion into an expression cassette are those which code for a DOXS and which confer on the host the ability to overproduce tocopherol.

The expression cassettes additionally comprise regulatory nucleic acid sequences which control the expression of the coding sequence in the host cell. In a preferred embodiment, an expression cassette comprises a promoter upstream, i.e. at the 5' end of the coding sequence, and a polyadenylation signal downstream, i.e. at the 3' end, and, where appropriate, further regulatory elements which are operatively linked to the coding sequence for the DOXS gene located in between. Operative linkage means sequential arrangement of promoter, coding sequence, terminator and, where appropriate, further regulatory elements in such a way that each of the regulatory elements can properly carry out its function in the expression of the coding sequence. The sequences which are preferred for the operative linkage, but are not restricted thereto, are targeting sequences to ensure subcellular localization in the apoplast, in the vacuole, in plastids, in the mitochondrion, in the endoplasmic reticulum (ER), in the cell nucleus, in elaioplasts or other compartments and translation enhancers such as the 5' leader sequence from tobacco mosaic virus (Gallie et al., Nucl. Acids Res. 15 (1987) 8693 - 8711).

For example, the plant expression cassette can be incorporated into the tobacco transformation vector pBinAR-Hyg. Fig. [lacuna] shows the tobacco transformation vectors pBinAR–Hyg with the 35S

promoter (A) and pBinAR–Hyg with the seed-specific promoter phaseolin 796 (B):

- HPT: hygromycin phosphotransferase
- OCS: octopine synthase terminator
- PNOS: nopaline synthase promoter
- also drawn in are those restriction cleavage sites which cut the vector only once.

Suitable promoters for the expression cassette are in principle all promoters able to control expression of foreign genes in plants. Preferably used is, in particular, a plant promoter or a promoter derived from a plant virus. The CaMV 35S promoter from cauliflower mosaic virus (Franck et al., Cell 21 (1980), 285-294) is particularly preferred. This promoter contains, as is known, different recognition sequences for transcriptional effectors which, in their totality, lead to permanent and constitutive expression of the inserted gene (Benfey et al., EMBO J. 8 (1989), 2195-2202).

The expression cassette may also contain a chemically inducible promoter by which expression of the exogenous DOXS gene in the plant can be controlled at a particular time. Promoters of this type, such as the PRP1 promoter (Ward et al., Plant. Mol. Biol. 22 (1993), 361-366), a promoter inducible by salicylic acid (WO 95/19443), a benzenesulfonamide-inducible (EP–A 388186), a tetracycline-inducible (Gatz et al., (1992) Plant J. 2, 397-404), an abscisic acid-inducible (EP–A 335528) and an ethanol- or cyclohexanone-inducible (WO 93/21334) promoter, inter alia, can be used.

Further particularly preferred promoters are those which ensure expression in tissues or parts of plants in which the biosynthesis of tocopherol or its precursors takes place. Particular mention should be made of promoters which ensure leaf-specific expression. Mention should be made of the promoter of cytosolic FBPase from potato or the ST-LSI promoter from potato (Stockhaus et al., EMBO J. 8 (1989) 2445 - 245).

It has been possible with the aid of a seed-specific promoter to express a foreign protein stably up to a content of 0.67% of the total soluble seed protein in the seeds of transgenic tobacco plants (Fiedler and Conrad, Bio/Technology 10 (1995), 1090-1094). The expression cassette can therefore contain, for example, a seed-specific promoter (preferably the phaseolin promoter (US 5504200), the USP (Baumlein, H. et al. Mol. Gen. Genet. (1991) 225 (3), 459 – 467) or LEB4 promoter (Fiedler and Conrad,

1995)), the LEB4 signal peptide, the gene to be expressed, and an ER retention signal. The construction of a cassette of this type is depicted diagrammatically by way of example in Figure 2.

An expression cassette is produced by fusing a suitable promoter to a suitable DOXS DNA sequence and, preferably, to a DNA which is inserted between promoter and DOXS DNA sequence and which codes for a chloroplast-specific transit peptide, and to a polyadenylation signal, by conventional recombination and cloning techniques as described, for example, in T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and in T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987).

Particularly preferred sequences are those which ensure targeting in the apoplast, in plastids, in the vacuole, in the mitochondrion, in the endoplasmic reticulum (ER) or, due to absence of appropriate operative sequences, ensure retention in the compartment of production, the cytosol (Kermode, Crit. Rev. Plant Sci. 15, 4 (1996), 285 - 423). Localization in the ER has proved particularly beneficial for the amount of protein accumulated in transgenic plants (Schouten et al., Plant Mol. Biol. 30 (1996), 781 - 792).

It is also possible to use expression cassettes whose DNA sequence codes for a DOXS fusion protein, where part of the fusion protein is a transit peptide which controls translocation of the polypeptide. Transit peptides specific for chloroplasts are particularly preferred, and these are eliminated enzymatically from the DOXS part after translocation of the DOXS gene into the chloroplasts. The particularly preferred transit peptide is derived from the plastidic transketolase (TK) or a functional equivalent of this transit peptide (e.g. the transit peptide of the small subunit of rubisco or ferredoxin-NADP oxidoreductase).

The inserted nucleotide sequence coding for a DOXS can be prepared by synthesis or be obtained naturally or comprise a mixture of synthetic and natural DNA constituents, and may consist of different heterologous DOXS gene sections from different organisms. In general, synthetic nucleotide sequences are produced with codons preferred by plants. These codons preferred by plants can be identified from codons with the highest protein frequency which are expressed in most plant

species of interest. For preparing an expression cassette it is possible to manipulate various DNA fragments in order to obtain a nucleotide sequence which expediently reads in the correct direction and is equipped with a correct reading frame. Adapters or linkers can be attached to the fragments for connecting the DNA fragments to one another.

It is possible and expedient for the promoter and terminator regions to be provided in the direction of transcription with a linker or polylinker which contains one or more restriction sites for inserting this sequence. As a rule, the linker has 1 to 10, usually 1 to 8, preferably 2 to 6, restriction sites. The linker generally has a size of less than 100 bp, frequently less than 60 bp, but at least 5 bp, inside the regulatory regions. The promoter may be both native or homologous and foreign or heterologous to the host plant. The expression cassette comprises in the 5'-3' direction of transcription the promoter, a DNA sequence which codes for a DOXS gene, and a region for termination of transcription. Various termination regions are interchangeable as desired.

It is furthermore possible to employ manipulations which provide appropriate restriction cleavage sites or delete the redundant DNA or restriction cleavage sites. It is possible in relation to insertions, deletions or substitutions, e.g. transitions and transversions, to use *in vitro* mutagenesis, primer repair, restriction or ligation. It is possible with suitable manipulations, e.g. restriction, chewing back or filling in overhangs for blunt ends, to provide complementary ends of the fragments for ligation.

It may be important for success according to the invention inter alia to attach the specific ER retention signal SEKDEL (Schouten, A. et al. Plant Mol. Biol. 30 (1996), 781 - 792), whereby the average level of expression is tripled or quadrupled. It is also possible to employ other retention signals which naturally occur with plant and animal proteins which are localized within the ER for constructing the cassette.

Preferred polyadenylation signals are plant polyadenylation signals, preferably those which essentially correspond to T-DNA polyadenylation signals from *Agrobacterium tumefaciens*, especially of gene 3 of the T-DNA (octopine synthase) of the Ti plasmid pTiACH5 (Gielen et al., EMBO J. 3 (1984) 835 ff) or functional equivalents.

An expression cassette may comprise, for example, a constitutive promoter (preferably the CaMV 35 S promoter), the LeB4 signal peptide, the gene to be expressed, and the ER retention signal. The ER retention signal preferably used is the amino acid sequence KDEL (lysine, aspartic acid, glutamic acid, leucine).

The fused expression cassette which codes for a DOXS gene is preferably cloned into a vector, for example pBin19, which is suitable for transforming Agrobacterium tumefaciens. Agrobacteria transformed with such a vector can then be used in a known manner for transforming plants, in particular crop plants, e.g. tobacco plants, by, for example, bathing wounded leaves or pieces of leaf in a solution of agrobacteria and then cultivating in suitable media. The transformation of plants by agrobacteria is dislosed inter alia by F.F. White, Vectors for Gene Transfer in Higher Plants; in Transgenic Plants, Vol. 1, Engineering and Utilization, edited by S.D. Kung and R. Wu, Academic Press, 1993, pp. 15-38. Transgenic plants containing a gene, integrated in the expression cassette, for expression of a DOXS gene can be regenerated in a known manner from the transformed cells of the wounded leaves or pieces of leaf.

For transformation of a host plant with a DNA coding for a DOXS, an expression cassette is incorporated as insertion into a recombinant vector whose vector DNA comprises additional functional regulatory signals, for example sequences for replication or integration. Suitable vectors are described inter alia in "Methods in Plant Molecular Biology and Biotechnology" (CRC Press), Chap. 6/7, pp. 71-119 (1993).

It is possible by using the recombination and cloning techniques cited above to clone the expression cassettes into suitable vectors which make their replication possible, for example in *E. coli*. Suitable cloning vectors are, inter alia, pBR332, pUC series, M13mp series and pACYC184. Binary vectors able to replicate both in *E. coli* and in agrobacteria are particularly suitable.

The invention further relates to the use of an expression cassette comprising a DNA sequence SEQ No. 1 or SEQ ID No. 3; SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 5; SEQ ID No. 1 or SEQ-ID No. 3 and SEQ-ID No. 7 or a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 5 and SEQ ID No. 7, or DNA sequences hybridizing with the latter for transforming plants, or cells, tissues or parts of plants. The aim of the use is preferably to

increase the tocopherol, vitamin K, chlorophyll and carotenoid contents of the plant.

It is moreover possible, depending on the choice of the promoter, for expression to take place specifically in the leaves, in the seeds, or other parts of the plant. The present invention further relates to such transgenic plants, to propagation material thereof and to cells, tissues or parts of the plants.

The expression cassette can in addition be employed for transforming bacteria, cyanobacteria, yeasts, filamentous fungi and algae with the aim of increasing tocopherol, vitamin K, chlorophyll and/or carotenoid production.

The transfer of foreign genes into the genome of a plant is referred to as transformation. In this connection, the described methods for transforming and regenerating plants from plant tissues or plant cells are utilized for transient or stable transformation. Suitable methods are protoplast transformation by polyethylene glycol-induced DNA uptake, the biolistic method using the gene gun - called the particle bombardment method, electroporation, incubation of dry embryos in DNA-containing solution, microinjection and gene transfer mediated by *Agrobacterium*. Said processes are described, for example, in B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, edited by S.D. Kung and R. Wu, Academic Press (1993) 128-143 and in Potrykus Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991) 205-225). The construct to be expressed is preferably cloned into a vector which is suitable for transforming *Agrobacterium tumefaciens*, for example pBin19 (Bevan et al., Nucl. Acids Res. 12 (1984) 8711).

Agrobacteria transformed with an expression cassette can likewise be used in a known manner for transforming plants, in particular crop plants such as cereals, corn, oats, soybean, rice, cotton, sugarbeet, canola, sunflower, flax, hemp, potato, tobacco, tomato, oilseed rape, alfalfa, lettuce and the various tree, nut and vine species, e.g. by bathing wounded leaves or pieces of leaf in a solution of agrobacteria and subsequently cultivating in suitable media.

Functionally equivalent sequences which code for a DOXS gene are sequences which, despite differing in nucleotide sequence, still have the required functions. Functional equivalents thus comprise naturally occurring variants of the sequences described herein, and artificial artificial nucleotide sequences obtained, for

example, by chemical synthesis and adapted to the codon usage of a plant.

A functional equivalent also means in particular natural or artificial mutations of an originally isolated sequence coding for a DOXS, which still show the required funtion. Mutations comprise substitutions, additions, deletions, transpositions or insertions of one or more nucleotide residues. Thus, the present invention also includes, for example, nucleotide sequences which are obtained by modifying the DOXS nucleotide sequence. The aim of such a modification may be, for example, to localize further the coding sequence present therein or else, for example, to insert further restriction enzyme cleavage sites.

Functional equivalents are also variants whose function is attenuated or enhanced by comparison with the initial gene or gene fragment.

Artificial DNA sequences are also suitable as long as they confer, as described above, the required property, for example of increasing the tocopherol content in the plant by overexpression of the DOXS gene in crop plants. Such artificial DNA sequences can be identified, for example, by back-translation of proteins which have been constructed by molecular modelling and have DOXS activity, or by *in vitro* selection. Particularly suitable coding DNA sequences are those which have been obtained by back-translation of a polypeptide sequence in accordance with the codon usage specific for the host plant. The specific codon usage can easily be established by a skilled worker familiar with plant genetic methods through computer analyses of other, known genes in the plant to be transformed.

Further suitable equivalent nucleic acid sequences which should be mentioned are sequences which code for fusion proteins, in which case a plant DOXS polypeptide or a functionally equivalent part thereof is a constituent of the fusion protein. The second part of the fusion protein can be, for example, another polypeptide with enzymatic activity, or an antigenic polypeptide sequence with whose aid it is possible to detect DOXS expression (e.g. myc tag or his tag). However, this is preferably a regulatory protein sequence, e.g. a signal or transit peptide which guides the DOXS protein to the required site of action.

However, the invention also relates to the expression products generated according to the invention, and to fusion proteins composed of a transit peptide and a polypeptide with DOXS

activity.

Increasing the tocopherol, vitamin K, chlorophyll and/or carotenoid content means for the purpose of the present invention the artificially acquired capability of increased activity in the biosynthesis of these compounds through functional overexpression of the DOXS gene in the plant compared with the plant which has not been genetically modified, for the duration of at least one plant generation.

The site of tocopherol biosynthesis is generally the leaf tissue so that leaf-specific expression of the DOXS gene is sensible. However, it is obvious that tocopherol biosynthesis need not be confined to the leaf tissue, but may also take place tissue-specifically in all other parts of the plant - for example in oilbearing seeds.

Constitutive expression of the exogenous DOXS gene is an additional advantage. However, on the other hand, inducible expression may also appear to be desirable.

The effectiveness of expression of the transgenically expressed DOXS gene can be determined, for example, *in vitro* by shoot meristem propagation. In addition, an alteration in the nature and level of the expression of the DOXS gene and its effect on tocopherol biosynthesis activity can be tested in glasshouse experiments on test plants.

The invention additionally relates to transgenic plants transformed with an expression cassette comprising the sequence SEQ ID No.1 or SEQ ID No. 3; SEQ ID No. 1 or SEQ ID No. 3 and SEQ No. 5; SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 7 or a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 and SEQ ID No. 5 and SEQ ID No. 7, or DNA sequences hybridizing with the latter, and transgenic cells, tissues, parts and propagation material of such plants. Particularly preferred in this connection are transgenic crop plants such as, for example, barley, wheat, rye, corn, oats, soybean, rice, cotton, sugarbeet, canola, sunflower, flax, hemp, potato, tobacco, tomato, oilseed rape, alfalfa, lettuce and the various tree, nut and vine species.

Plants for the purpose of the invention are mono- and dicotyledonous plants or algae.

In order to be able to find efficient DOXS inhibitors, it is necessary to provide suitable test systems with which inhibitor/enzyme binding studies can be carried out. For this purpose, for

example, the complete cDNA sequence of DOXS from Arabidopsis is cloned into an expression vector (pQE, Qiagen) and overexpressed in *E. coli*.

The DOXS protein expressed using the expression cassette is particularly suitable for finding inhibitors specific for DOXS.

For this purpose, DOXS can be employed, for example, in an enzyme assay in which the activity of DOXS is determined in the presence and absence of the active substance to be tested. Comparison of the two activity determinations allows qualitative and quantitative information to be obtained about the inhibitory behavior of the active substance to be tested. Methods for DOXS activity determination are described (Putra et. al., Tetrahedron Letters 39 (1998), 23-26; Sprenger et al., PNAS 94 (1997), 12857-12862).

The test system according to the invention can be used to examine rapidly and simply a large number of chemical compounds for inhibitory properties. The method allows reproducible selection, from a large number of substances, specifically of those with high activity in order subsequently to carry out with these substances further, more intensive tests familiar to the skilled worker.

It is possible in principle by overexpression of the gene sequence SEQ ID NO: 1 or SEQ ID NO: 3 coding for DOXS in a plant to achieve increased resistance to DOXS inhibitors. The invention likewise relates to transgenic plants produced in this way.

The invention further relates to:

- A process for transforming a plant, which comprises introducing an expression cassette comprising a DNA sequence SEQ ID No. 1 or SEQ ID No. 3 or a DNA sequence hybridizing with the latter into a plant cell, into callus tissue, a whole plant or protoplasts of plants.

- The use of a plant for producing plant DOXS.

- The use of the expression cassette comprising a DNA sequence SEQ ID No. 1 or SEQ ID NO. 3 or a DNA sequence hybridizing with the latter for producing plants with increased resistance to DOXS inhibitors by enhanced expression of a DNA

sequence SEQ ID No. 1 or SEQ ID NO. 3 or a DNA sequence hybridizing with the latter.

- The use of the DNA sequence SEQ ID No. 1 or SEQ ID NO. 3 or of a DNA sequence hybridizing with the latter for producing plants with increased tocopherol, vitamin K, chlorophyll and/or carotenoid content by expression of a DOXS DNA sequence in plants.

- The use of the expression cassette comprising a DNA sequence SEQ ID No. 1 or SEQ ID NO: 3 or a DNA sequence hybridizing with the latter for producing a test system for identifying DOXS inhibitors.

The invention is illustrated by the examples which now follow, but is not confined to these:

General cloning methods

The cloning steps carried out for the purpose of the present invention, such as restriction cleavages, agarose gel electrophoresis, purification of DNA fragments, transfer of nucleic acids to nitrocellulose and nylon membranes, linkage of DNA fragments, transformation of E. coli cells, cultivation of bacteria, replication of phages and recombinant DNA sequence analysis were carried out as described in Sambrook et al. (1989) Cold Spring Harbor Laboratory Press; ISBN 0-87969-309-6).

The bacterial strains (*E. coli*, XL-I Blue) used below were purchased from Stratagene. The agrobacterium strain used for plant transformation (*Agrobacterium tumefaciens*, C58C1 with the plasmid pGV2260 or pGV3850kann) has been described by Deblaere et al. in (Nucl. Acids Res. 13 (1985) 4777). Alternative possibilities are also to employ the agrobacterium strain LBA4404 (Clontech) or other suitable strains. Vectors which can be used for cloning are pUC19 (Yanish-Perron, Gene 33 (1985), 103-119) pBluescript SK- (Stratagene), pGEM-T (Promega), pZerO (Invitrogen), pBin19 (Bevan et al., Nucl. Acids Res. 12 (1984), 8711-8720) and pBinAR (Höfgen and Willmitzer, Plant Science 66 (1990), 221-230).

Recombinant DNA sequence analysis

Recombinant DNA molecules were sequenced using a Licor laser fluorescence DNA sequencer (marketed by MWG Biotech, Ebersbach)

using the Sanger method (Sanger et al., Proc. Natl. Acad. Sci. USA 74 (1977), 5463-5467).

Example 1

Production of the Arabidopsis thaliana DOXS transformation constructs

The Arabidopsis thaliana DOXS gene was cloned as described in Mandel et al. (1996) as complete cDNA into the vector pBluescript KS- (Stratagene).

To produce overexpression constructs, a 2.3 kb fragment (designated F-23-C) was isolated via the pBluescript KS- HincII (blunt-end) and SacI cleavage sites. This sequence contains the complete DOXS cDNA from the ATG start codon to the EcoRII cleavage site located 80 bp downstream of the stop codon. This fragment was cloned via the SmaI (blunt-end) and SacI cleavage sites into the pBIN19 vector (Figure 3) (Bevan et al., (1980) which contains the 35S promoter of cauliflower mosaic virus (Franck et al., Cell 21(1), 285-294 (1980)) arranged three times in sequence.

To produce antisense constructs, a region of the 3' end of the cDNA (called F-23-C antisense) was cloned into the abovementioned pBIN19-3X35S vector. Part of the 5' region of the DOXS cDNA in pBluescript KS- was digested via HincII and the DOXS-internal BglII cleavage site, and the resulting fragment was removed. (Figure 4). The BglII cleavage site was filled in by the Klenow fill-in reaction (Klenow polymerase; Roche; after reaction according to manufacturer's protocol) so that a blunt end was produced. The ends which were now compatible (BglII blunt end and HinclII were ligated. The 3' region of the DOXS cDNA was then cloned via KpnI and XbaI (both cleavage sites are located in the polylinker of pBluescript KS-5' and 3' of the DOXS cDNA) in antisense orientation into the pBIN19 vector described above in antisense orientation.

Transformations of Arabidopsis thaliana plants with the constructs described above using Agrobacterium tumefaciens took place by the vacuum infiltration method (Bent et al., Science 265 (1994), 1856-1860). Several independent transformands were isolated for each construct. Each letter (see Table 1) denotes an independent transformed line. Plants from the T1 generation obtained therefrom were examined for homo- or heterozygosity. Several plants from each line were crossed in order to carry out a segregation analysis. The number in Table 1 corresponds to the

individual plant selected for further analyses. Both homo- and heterozygous lines were obtained. The segregation analysis of the resulting lines is shown in Table 1 below:

Table 1. Segregation analysis of the transgenic DOXS T2 plants

LINES	SEGREGATION
A9	75%
A19	100%
B11	75%
B4	100%
C2	100%
D3	75%
D17	100%
E9	75%
E14	100%
F9	75%
F14	100%

Example 2

Isolation of genomic DNA of the bacterium Escherichia coli XL1 Blue

A culture of Escherichia coli XL1 Blue was grown in 300 ml of Luria broth medium at 37°C for 12 hours. The genomic DNA of the bacterium was isolated from this culture by first pelleting it at 5 000 revolutions in a Sorvall RC50 fuge. The pellet was then resuspended in 1/30 of the volume of the original culture of lysis buffer (25 mM EDTA, 0.5% SDS; 50 mM Tris HCl, pH 8.0). An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and incubated at 70 degrees for 10 minutes. The aqueous phase was then separated from the phenolic in a Heraeus floor centrifuge at 3 500 rev for 15 minutes. The aqueous supernatant was mixed with 2.5 volumes of ethanol and 1/10 volume of 8 M lithium chloride, and the nucleic acids were precipitated at room temperature for 10 minutes. The pellet was then taken up in 400 µl of TE/RNAse and incubated at 37 degrees for 10 minutes. The solution was again shaken with one volume of phenol/ chloroform/isoamyl alcohol (25:24:1), and the supernatant was precipitated with 2.5 volumes of ethanol and 1/10 volume of 8 M lithium chloride. The pellet was then washed with 80% ethanol and taken up in 400 µl of TE/RNAse.

Example 3

Isolation of the DOXS from E. coli

Oligonucleotides for a PCR were derived from the DOXS DNA sequence (Acc. Number AF035440), and a BamHI restriction cleavage site was attached to them at the 5' end, and an XbaI or another BamHI restriction cleavage site was attached to them at the 3' end. The oligonucleotide at the 5' end comprises the sequence 5'-ATGGATCC*ATGAGTTTT-GATATTGCCAAATAC*-3' (nucleotides 1-24 of the DNA sequence; in italics) starting with the ATG start codon of the gene, and the oligonucleotide at the 3' end comprises the sequence 5'-ATTCTAGA*TTATGCCAGCCAGGCCTTG*-3' or 5'-ATGGATCC*TTATGCCAGCCAGGCCTTG*-3' (nucleotides 1845-1863 of the reverse complementary DNA sequence; in italics) starting with the stop codon of the gene. The PCR reaction with the two BamHI-containing oligonucleotides was carried out with Pfu polymerase (Stratagene GmbH, Heidelberg) in accordance with the manufacturer's information. 500 ng of the genomic DNA from E. coli were employed as template. The PCR program was as follows:

5 cycles: 4 sec 94°C, 30 sec 52°C, 2 min 72°C;
5 cycles: 4 sec 94°C, 30 sec 48°C, 2 min 72°C;
25 cycles: 4 sec 94°C, 30 sec 44°C, 2 min 72°C

The fragment was purified using a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script (Stratagene GmbH, Heidelberg). The correctness of the sequence was established by sequencing. The fragment was BamHI isolated from the PCR-Script vector and ligated into a correspondingly cut Bin19 vector which additionally contains the transit peptide of potato transketalase downstream of the CaMV 35S as promoter. The transit peptide ensures plastidic localization. The constructs are depicted in Figure 5 and 6, and the fragments have the following significance:

Fragment A (529 bp) comprises the 35S promoter of cauliflower mosaic virus (nucleotides 6909 to 7437 of cauliflower mosaic virus). Fragment B (259 bp) comprises the transit peptide of transketolase. Fragment E comprises the DOXS gene. Fragment D (192 bp) comprises the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) to terminate transcription.

The PCR reaction with the 5'-BamHI and 3'-XbaI-containing oligonucleotides was carried out with Taq polymerase (Takara, Sosei Co., Ltd.) in accordance with the manufacturer's information. 500 ng of the genomic DNA from E. coli were employed as template. The PCR program was as follows:

5 cycles: 4 sec 94°C, 4 sec 50°C, 2 min 30°C
5 cycles: 4 sec 94°C, 30 sec 46°C, 2 min 68°C
25 cycles: 4 sec 94°C, 30 sec 42°C, 2 min 68°C

The fragment was purified using a Gene-Clean kit and ligated into the vector pGemT (Promega GmbH, Mannheim). It was cloned as BamHI/XbaI fragment into a correspondingly cut pBin19AR vector downstream of the CaMV 35S promoter. The sequence was checked by sequencing (SEQ-ID No. 3). This revealed two non-conservative base exchanges which, compared with the published sequence, lead to a change in amino acid 152 (asparagine) to valine and amino acid 330 (cysteine) to tryptophan.

Example 4

Detection of increased amounts of DOXS RNA in transgenic plants

Total RNA from 15-day old seedlings of various transgenic lines possessing the DOXS overexpression construct was extracted by the method of Logeman et al., Anal. Biochem. 163, 16-20 (1987), fractionated in a 1.2% agarose gel, transferred to filters and hybridized with a 2.1 kb long DOXS fragment as probe (Figure 7).

Example 5

Detection of increased amounts of DOXS protein in transgenic plants

Total protein (Figure 8) from 15-day old seedlings of various independent transgenic plants possessing the DOXS overexpression construct was isolated and detected in a Western analysis using a polyclonal anti-DOXS antibody (IgG) (Figure 9).

Example 6

Measurement of the carotenoid and chlorophyll contents

The total amounts of carotenoids and chlorophylls were determined as described by Lichtenthaler and Wellburn (1983) using 100% acetone extracts. The results of the multiple measurements of the

transgenic lines possessing the DOXS overexpression construct are shown in Table 2 below.

Table 2: Total carotenoid and chlorophyll contents of transgenic DOXS lines

LINE	% TOTAL CHLOROPHYLLS	% TOTAL CAROTENOIDS
cla1 mutant	5	5
Wild type	100	100
B-4	86	89
B-11	84	90
C-2	98	107
D-3	128	135
D-17	136	149
E-14	121	139
F-7	80	90
F-14	85	107

Example 7

Transformation of oilseed rape

The production of transgenic oilseed rape plants is based on a protocol of Bade, JB and Damm, B (in Gene, Transfer to Plants, Potrykus, I. and Spangenberg, G., eds, Springer Lab Manual, Springer Verlag, 1995, 30-38), in which the composition of the media used are also stated. The transformations took place with the agrobacterium strain LBA4404 (Clontech). The binary vectors used were the pBIN19 constructs with the complete DOXS cDNA already described above. The NOS terminator sequence in these pBIN vectors was replaced by the OCR terminator sequence. Brassica napus seeds were surface-sterilized with 70% (v/v) ethanol, washed in H_2O at 55°C for 10 min, incubated in 1% strength hypochlorite solution (25% v/v Teepol, 0.1% v/v Twenn 20) for 20 min and washed six times with sterile H_2O for 20 min each time. The seeds were dried on filter paper for three days and 10-15 seeds were induced to germinate in a glass flask with 15 ml of germination medium. The roots and apices were removed from several seedlings (about 10 cm in size), and the remaining hypocotyls were cut into pieces about 6 mm long. The approx. 600 explants obtained in this way are washed with 50 ml of basal medium for 30 min and transferred into a 300 ml flask. After addition of 100 ml of callus induction medium, the cultures were incubated at 100 rpm for 24 h.

An overnight culture of the agrobacterium strain was set up in LB with kanamycin (20 mg/l) at 29°C, and 2 ml of this were incubated in 50 ml of LB without kanamycin at 29°C for 4 h until the OD_{600} was 0.4-0.5. After pelleting of the culture at 2 000 rpm for 25 min, the cell pellet was resuspended in 25 ml of basal medium. The concentration of the bacteria in the solution was adjusted to an OD600 of 0.3 by adding further basal medium.

The callus induction medium was removed from the oilseed rape explants using sterile pipettes, 50 ml of agrobacterium solution were added and, after cautious mixing, incubated for 20 min. The agrobacteria suspension was removed, the oilseed rape explants were washed with 50 ml of callus induction medium for 1 min and then 100 ml of callus induction medium were added. The cocultivation was carried out on a rotary shaker at 100 rpm for 24 h. The cocultivation was stopped by removing the callus induction medium, and the explants were washed twice for 1 min each time with 25 ml and twice for 60 min with 100 ml each time of washing medium at 100 rpm. The washing medium with the explants was transferred into 15 cm Petri dishes, and the medium was removed using sterile pipettes. For regeneration, in each case 20-30 explants were transferred into 90 mm Petri dishes which contained 25 ml of shoot-induction medium with kanamycin. The Petri dishes were sealed with 2 layers of Leukopor and incubated at 25°C and with 2000 lux in 16/8 H photoperiods. The calli which developed was transferred every 12 days to fresh Petri dishes with shoot-induction medium. All further steps for regenerating whole plants ~~were~~ carried out as described by Bade, J.B. and Damm, B. (in Gene Transfer to Plants, Potrykus, I. and Spangenberg, G.,eds, Springer Lab Manual, Springer Verlag, 1995, 30-38).

Example 8

Increasing tocopherol biosynthesis in oilseed rape

The DOXS cDNA (SEQ-ID No. 1) was provided with a CaMV 35S promoter and over-expressed in oilseed rape using the 35S promoter. In parallel with this, the seed-specific promoter of the phaseolin gene was used in order specifically to increase the tocopherol content in the rapeseed. Oilseed rape plants transformed with the corresponding constructs were grown in a glasshouse. The α-tocopherol content of the whole plant and of the seeds of the plant was then determined. In all cases, the α-tocopherol concentration was increased by comparison with the untransformed plant.

Example 9

Detection of the expression of DOXS from E. coli in transgenic tobacco plants

Leaf disks with a diameter of 0.9 cm were taken from completely unfolded leaves of plants containing the construct pBinAR HPPD-DOXS, and were frozen in liquid nitrogen. The leaf material was homogenized in an HEPES-KOH buffer containing proteinase inhibitors, and the protein concentration was determined from the extract using the Bio-Rad protein assay in accordance with the manufacturer's information. 45 µg of protein from each extract were mixed with one volume of loading buffer (Laemmli, 1970) and incubated at 95°C for 5 min. The proteins were then fractionated on a 12.5 percent SDS-PAGE gel. The proteins were then transferred by means of semi-dry electroblots to Porablot membrane (Machery und Nagel). Detection of the DOXS protein took place using a rabbit antibody against E. coli DOXS. The color reaction is based on the binding of a secondary antibody and of an alkaline phosphatase which converts NBT/BCIP into a dye. Secondary antibody and alkaline phosphatase were obtained from Pierce, and the procedure was in accordance with the manufacturer's information.

Figure 10 shows the detection of the DOXS protein in leaves of transgenic plants. 1: marker; 2: plant 10; 3:62; 4: 63; 5: 69; 7:71; 8:112; 9:113; 10:116; 11:WT1; 12:WT2; 13:100 ng of recombinant protein; 14:50 ng of recombinant protein; 15: 10 ng of recombinant protein.

Example 10

Cloning of an HPPD gene from Streptomyces avermitilis U11864

Isolation of genomic DNA of the bacterium Streptomyces avermitilis U11864:

A culture of Streptomyces avermitilis U11864 was grown in 300 ml of YEME medium (5 g of malt extract, 2 g of yeast extract, 2 g of glucose) at 28°C for 96 h. The genomic DNA of the bacterium was isolated from this culture by pelleting it initially at 5000 rev in a Sorvall RC5C fuge. The pellet was then resuspended in 1/30 of the volume of lysis buffer (25 mM EDTA, 0.5% SDS, 50 mM Tris-HCl, pH 8.0). An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and incubated at 70°C for 10 minutes. The aqueous phase was then separated from the phenolic in a Heraeus floor centrifuge at 3 500 rev for 15 minutes. The aqueous

supernatant was mixed with 2.5 volumes of ethanol in 1/10 volume of 8 M lithium chloride, and the nucleic acids were precipitated at room temperature for 10 minutes. The pellet was then taken up in 400 µl of TE/RNAse and incubated at 37 degrees for 10 minutes. The solution was again shaken with one volume of phenol/chloroform/isoamyl alcohol (25:24:1), and the supernatant was precipitated with 2.5 volumes of ethanol and 1/10 volume of 8 M lithium chloride. The pellet was then washed with 80% ethanol and taken up in 400 µl of TE/RNAse.

Oligonucleotides were derived for a PCR from the DNA sequence of the HPPD from Streptomyces avermitilis (Denoya et al., 1944; Acc. Number U11864), and a BamHI restriction cleavage site was attached to the 5' end of them and an XbaI restriction cleavage site was attached at the 3' end of them. The oligonucleotide at the 5' end comprises the sequence 5'-GG*ATCCAGCGGACAAGCCAAC*-3' (37 to 55 bases distant from the ATG in the 5' direction; in italics), and the oligonucleotide at the 3' end comprises the sequence 5'-TCTAGA*TTATGCCAGCCAGGCCTTG*-3' (nucleotides 1845-1863 of the reverse complementary DNA sequence; in italics).

The PCR reaction was carried out with Pfu polymerase (Stratagene GmbH, Heidelberg) in accordance with the manufacturer's information. 400 ng of the genomic DNA was employed as pattern. The PCR program was as follows:

5 cycles: 4 sec 94°C, 30 sec 54°C, 2 min 72°C
5 cycles: 4 sec 94°C, 30 sec 52°C, 2 min 72°C
25 cycles: 4 sec 94°C, 30 sec 50°C, 2 min 72°C

The fragment was purified by means of a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script (Stratagene GmbH, Heidelberg). The correctness of the sequence was checked by sequencing. This revealed that the isolated gene codes for an additional amino acid. It contains the three bases TAC (coding for tyrosine) in front of nucleotide N429 in the quoted sequence (Denoya et al., 1994).

The fragment was isolated by a BamHI and XbaI digestion from the vector and ligated into a correspondingly cut Bin19AR vector downstream of the CaMV 35S promoter for expression of the gene in the cytosol. The gene was isolated as BamHI fragment from the same PCR-Script vector and was ligated into a correspondingly cut pBin19 vector which additionally comprises the transit peptide of the potato plastidic transketolase downstream of the CaMV 35S promoter. The transit peptide ensures the plastidic localization.

The constructs are depicted in Figure 11 and 12, and the fragments have the following significance:

Fragment A (529 bp) comprises the 35S promoter of cauliflower mosaic virus (nucleotides 6909 to 7437 of cauliflower mosaic virus). Fragment B (259 bp) comprises the transit peptide of transketolase. Fragment C comprises the HPPD gene. Fragment D (192 bp) comprises the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen, J. et al., EMBO J. 3 (1984), 835-846) to terminate transcription.

Example 11

Production of constructs for transformation of plants with DOXS and HPPD DNA sequences

To produce plants which are transgenic for DOXS and HPPD, a binary vector which contains both gene sequences was manufactured (Figure 13). The DOXS and HPPD gene sequences were each cloned as BamHI fragments as described in Example 3 and 10. The vector pBinAR-Hyg contains the 35S promoter of cauliflower mosaic virus and the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription. The pBinAR-Hyg vector confers on plants resistance to the antibiotic hygromycin and is therefore suitable for superinfection of plants with kanamycin resistance.

To clone the HPPD in vectors which additionally contain another cDNA, oligonucleotides were derived for a PCR and had a BamHI restriction cleavage site attached at the 5' end and at the 3' end. The oligonucleotide at the 5' end comprises the sequence 5'-GG*ATCCTCCAGCGGACAAGCCAAC*-3' (nucleotides 37 to 55 distant from the ATG in the 5' direction; in italics), and the oligonucleotide at the 3' end comprises the sequence 5'-ATGGATCC*CGCGCCGCCTACAGGTTG*-3' (terminating at base pair 1140 of the coding sequence, starting 8 base pairs 3' from the TAG stop codon; in italics). The PCR reaction was carried out with Tli polymerase (Promega GmbH, Mannheim) in accordance with the manufacturer's information. 10 ng of the plasmid pBinAR-HPPD were employed as template. The PCR program was as follows:

5 cycles: 94°C 4 sec, 68°C 30 sec, 72°C 2 min
5 cycles: 94°C 4 sec, 64°C 30 sec, 72°C 2 min
25 cycles: 94°C 4 sec, 60°C 30 sec, 72°C 2 min

The fragment was purified using a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script (Stratagene GmbH, Heidelberg). The correctness of the sequence was checked by sequencing. It was cut as BamHI fragment out of the vector PCR-Script and ligated into a correspondingly cut pBinAR vector which additionally contains the transit peptide of transketolase for introducing the gene product into the plastids. The result was the plasmid pBinAR-TP-HPPD (Figure 12).

For the cloning, the 35S promoter, the transketolase transit peptide, the HPPD gene and the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al. 1984) for termination of transcription was isolated from the plasmid pBinAR-TP-HPPD by PCR. A HindIII cleavage site was attached in each case to the oligonucleotides for the promoter and the terminator. The sequence of the oligonucleotide which anneals onto the 5' region of the promoter (in italics) is 5'-ATAAGCTT*CATGGAGTCAAA-GATTCAAATAGA*-3', and that of the oligonucleotide which anneals onto the termination sequence (in italics) is 5'-ATAAGCTT*GGACAATCAGTAAATTGAACGGAG*-3'. The resulting fragment was purified using a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script from Stratagene GmbH, Heidelberg. The correctness of the sequence was checked by sequencing. It was transferred as HindIII fragment from this PCR-Script vector into the correspondingly cut vector pBin19 (Bevan, 1984, Nucleic Acids Res. 12, 8711-8721).

The 35S promoter, the transketolase transit peptide, the DOXS gene and the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription was isolated by PCR from the plasmid pBinAR-TP-DOXS. An EcoRI cleavage site was attached to each of the oligonucleotides for the promoter and terminator sequence. The sequence of the oligonucleotide which anneals onto the promoter (in italics) is 5'-ATGAATTC*CATGGAGTCAAAGATTCAAATAGA*-3', and that of the oligonucleotide which anneals onto the terminator sequence (in italics) is 5'-ATGAATTC*GGACAATCAGTAAATTGAA-CGGAG*-3'. The fragment was purified using a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script (Stratagene GmbH, Heidelberg). The correctness of the sequence was checked by sequencing (SEQ ID No. 3). It was transferred as EcoRI fragment from the PCR-Script vector into the correspondingly cut vector pBin19 (Bevan, 1984).

It was transferred as XbaI fragment from the PCR-Script vector into the correspondingly cut vector which, as described above, already contains the HPPD sequence. The result was the construct pBinAR-HPPD-DOXS (Figure 13), whose fragments have the following significance:

Fragment A (529 bp) comprises the 35S promoter of the cauliflower mosaic virus (nucleotides 6909 to 7437). Fragment B comprises the transit peptide of plastidic transketolase. Fragment C comprises the HPPD gene. Fragment D comprises the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription. Fragment E comprises the DOXS gene.

Example 12

Production of transgenic tobacco plants
(Nicotiana tabacum L. cv. Samsun NN)

Transgenic tobacco plants having an altered prenyl lipid content were produced by transforming tobacco leaf disks with DOXS and HPPD sequences. To transform tobacco plants, 10 ml of an Agrobacterium tumefaciens overnight culture which had grown under selection were centrifuged, the supernatant was discarded and the bacteria were resuspended in the same volume of antibiotic-free medium. Leaf disks from sterile plants (diameter about 1 cm) were bathed in this bacterial suspension in a sterile Petri dish. The leaf disks were then placed on MS medium (Murashige and Skoog, Physiol. Plant (1962) 15, 473) with 2% sucrose and 0.8% Bacto agar in Petri dishes. After incubation in the dark at 25°C for 2 days, they were transferred to MS medium with 100 mg/l kanamycin, 500 mg/l Claforan, 1 mg/l benzylaminopurine (BAP), 0.2 mg/l naphthylacetic acid (NAA), 1.6% glucose and 0.8% Bacto agar, and the cultivation was continued (16 hours of light/ 8 hours of dark). Growing shoots were transferred to hormone-free MS medium with 2% sucrose, 250 mg/l Claforan and 0.8% Bacto agar.

Example 13

Production of transgenic oilseed rape plants (Brassica napus)

The production of transgenic oilseed rape plants having an altered prenyl lipid content was based on a protocol by Bade, J.B. and Damm, B. (in Gene Transfer to Plants, Potrykus, I. and Spangenberg, G., eds, Springer Lab Manual, Springer Verlag,

1995, 30-38), in which the compositions of the media and buffers used are also indicated.

The transformations took place with the Agrobacterium tumefaciens strain LBA4404 (Clontech GmbH, Heidelberg). The binary vectors used were the binary constructs already described above with the total cDNAs of DOXS and HPPD. In all the binary vectors used here, the NOS terminator sequence was replaced by the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription. Brassica napus seeds were surface-sterilized with 70% (v/v) ethanol, washed in H_2O at 55°C for 10 min, incubated in 1% strength hypochlorite solution (25% v/v Teepol, 0.1% v/v Tween 20) for 20 min and washed six times with sterile H_2O for 20 min each time. The seeds were dried on filter paper for three days and 10-15 seeds were induced to germinate in a glass flask with 15 ml of germination medium. The roots and apices were removed from several seedlings (about 10 cm in size), and the remaining hypocotyls were cut into pieces about 6 mm long. The approx. 600 explants obtained in this way are washed with 50 ml of basal medium for 30 min and transferred into a 300 ml flask. After addition of 100 ml of callus induction medium, the cultures were incubated at 100 rpm for 24 h.

An overnight culture of the agrobacterium strain was set up in Luria Broth medium with kanamycin (20 mg/l) at 29°C, and 2 ml of this were incubated in 50 ml of Luria Broth medium without kanamycin at 29°C for 4 h until the OD_{600} was 0.4-0.5. After pelleting of the culture at 2 000 rpm for 25 min, the cell pellet was resuspended in 25 ml of basal medium. The concentration of the bacteria in the solution was adjusted to an OD_{600} of 0.3 by adding further basal medium.

The callus induction medium was removed from the oilseed rape explants using sterile pipettes, 50 ml of agrobacterium solution were added and, after cautious mixing, incubated for 20 min. The agrobacteria suspension was removed, the oilseed rape explants were washed with 50 ml of callus induction medium for 1 min and then 100 ml of callus induction medium were added. The cocultivation was carried out on a rotary shaker at 100 rpm for 24 h. The cocultivation was stopped by removing the callus induction medium, and the explants were washed twice for 1 min each time with 25 ml and twice for 60 min with 100 ml each time of washing medium at 100 rpm. The washing medium with the explants was transferred into 15 cm Petri dishes, and the medium was removed using sterile pipettes.

For regeneration, in each case 20-30 explants were transferred into 90 mm Petri dishes which contained 25 ml of shoot-induction medium with kanamycin. The Petri dishes were sealed with 2 layers of Leukopor and incubated at 25°C and with 2 000 lux in photoperiods of 16 hours of light/8 hours of dark. The calli which developed were transferred every 12 days to fresh Petri dishes with shoot-induction medium. All further steps for regenerating whole plants were carried out as described by Bade, J.B. and Damm, B. (in Gene Transfer to Plants, Potrykus, I. and Spangenberg, G.,eds, Springer Lab Manual, Springer Verlag, 1995, 30-38).

Example 14

Increasing tocopherol biosynthesis in oilseed rape

The cDNA of DOXS (SEQ-ID No. 3) and of HPPD (SEQ-ID No. 5) was provided with a CaMV35S promoter and overexpressed in oilseed rape using the 35S promoter. In parallel with this, the seed-specific promoter of the phaseolin gene was used in order specifically to increase the tocopherol content in the rape seed. Oilseed rape plants transformed with the corresponding constructs were grown in a glasshouse. The α-tocopherol content of the whole plant and of the seeds of the plant was then determined. In all cases, the α-tocopherol concentration was increased by comparison with the untransformed plant.

Example 15

Cloning of a GGPPOR gene from Arabidopsis thaliana

Isolation of total RNA from completely unfolded leaves of Arabidopsis thaliana:

Completely unfolded leaves of Arabidopsis thaliana were harvested and frozen in liquid nitrogen. The material was then powdered in a mortar and taken up in Z6 buffer (8 M guanidium hydrochloride, 20 mM MES, 20 mM EDTA pH 7.0). The suspension was transferred into reaction vessels and shaken with one volume of phenol/ chloroform/isoamyl alcohol 25:24:1). After centrifugation at 15 000 rpm for 10 minutes, the supernatant was transferred into a new reaction vessel, and the RNA was precipitated with 1/20 volumes of 1N acetic acid and 0.7 volume of ethanol (absolute). After renewed centrifugation, the pellet was first washed with 3M sodium acetate solution and, after a further centrifugation, in 70% ethanol. The pellet was then dissolved in

DEPC water, and the RNA concentration was determined by photometry.

Production of cDNA from total RNA from completely unfolded leaves of A. thaliana:

20 µg of total RNA were initially mixed with 3.3 µl of 3M sodium acetate solution and 2 µl of 1M magnesium sulfate solution and made up to a final volume of 100 µl with DEPC water. 1 µl of RNase-free DNase (Boehringer Mannheim) was added to this and incubated at 37°C for 45 min. After removal of the enzyme by shaking with phenol/chloroform/isoamyl alcohol, the RNA was precipitated with ethanol, and the pellet was taken up in 100 µl of DEPC water. 2.5 µg of RNA from this solution were transcribed into cDNA by means of a cDNA kit (Gibco, Life Technologies).

Oligonucleotides were derived for a PCR from the geranylgeranyl-pyrophosphate oxidoreductase DNA sequence (Keller et al., Eur. J. Biochem. (1998)251(1-2), 413-417); Accession Number Y14044), and a BamHI restriction cleavage site was attached at the 5' end of these and a SalI restriction cleavage site was attached at the 3' end. The oligonucleotide at the 5' end comprises the sequence 5'-ATGGATCC*ATGGCGACGACGGTTACACTC*-3' starting with the first codon of the cDNA (in italics), and the oligonucleotide at the 3' end comprises the sequence 5'-ATGTCGAC*GTGATGATAGATTACTAACAGAC*-3' starting with base pair 1494 of the cDNA sequence (in italics).

The PCR reaction was carried out with Pfu polymerase from Stratagene GmbH, Heidelberg in accordance with the manufacturer's information. 1/8 of a volume of the cDNA was employed as template (equivalent to 0.3 µg of RNA). The PCR program was as follows:

5 cycles: 94°C for 4 sec, 48°C for 30 sec, 72°C for 2 min
5 cycles: 94°C for 4 sec, 46°C for 30 sec, 72°C for 2 min
25 cycles: 94°C for 4 sec, 44°C for 30 sec, 72°C for 2 min

The fragment was purified using a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script from Stratagene GmbH, Heidelberg. The correctness of the fragment was checked by sequencing (SEQ ID No. 7). The gene was cloned by means of the restriction cleavage sites attached to the sequence by the primers as BamHI/SalI fragment into the correspondingly cut vector BinAR-Hyg. The latter contains the 35S promoter of cauliflower mosaic virus and the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., EMBO J. 3 (1984), 835-846) for termination of transcription. The plasmid

confers on plants resistance to the antibiotic hygromycin and is thus suitable for superinfection of plants with kanamycin resistance. Since the plastid transit peptide of GGPPOR was also cloned, the protein ought to be transported into the plastids in transgenic plants. The construct is depicted in Figure 14. The fragments have the following significance:

Fragment A (529 bp) comprises the 35S promoter of cauliflower mosaic virus (nucleotides 6909 to 7437 of cauliflower mosaic virus). Fragment D comprises the polyadenylation signal of gene 3 of the T DNA of Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription. Fragment F comprises the gene of GGPPOR including the intrinsic plastid transit sequence.

Example 16

Production of constructs for transformation of plants with DOXS and GGPPOR sequences

To produce plants which are transgenic for DOXS and GGPPOR, a binary vector comprising both gene sequences was manufactured (Figure 15). The GGPPOR gene with the intrinsic plastidic localization sequence was cloned (as described in Example 15) as BamHI/SalI fragment into the correspondingly cut vector pBinAR-Hyg. The DOXS gene was cloned as BamHI fragment as described in Example 3. The vector pBinAR-Hyg contains the 35S promoter of cauliflower mosaic virus and the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription. This plasmid confers on plants resistance to the antibody hygromycin and is thus suitable for superinfection of plants with kanamycin resistance.

The 35S promoter, the transketolase transit peptide, the DOXS gene and the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription was isolated from the plasmid pBinAR-TP-DOXS by PCR. An EcoRI cleavage site was attached in each case to the oligonucleotides for the promoter and the terminator sequence. The sequence of the oligonucleotide which anneals onto the promoter (in italics) is 5'-ATGAATTC*CATGGAGTCAAAGATTCAAATAGA*-3', and that of the oligonucleotide which anneals onto the terminator sequence (in italics) is 5'-ATGAATTC*GGACAATCAGTAAATTGAACGGAG*-3'. The fragment was purified using a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script from Stratagene GmbH, Heidelberg. The correctness of the sequence was checked by

sequencing. It was transferred from the PCR-Script vector as EcoRI fragment into the correspondingly cut vector pBin19 (Bevan, Nucleic Acids Res. 12 (1984), 8711-8721).

The 35S promoter, the GGPPOR gene and the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription was isolated from the plasmid pBinARHyg-GGPPOR by PCR. An XbaI cleavage site was attached in each case to the oligonucleotides for the promoter and the terminator. The sequence of the oligonucleotide which anneals onto the promoter (in italics) is 5'-ATTCTAGA*CATGGAGTCAAA-GATTCAAATAGA*-3', and that of the oligonucleotide which anneals onto the terminator sequence (in italics) is 5'-ATTCTAGA*GGACAA-TCAGTAAATTGAACGGAG*-3'. The fragment was purified using a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information onto the vector PCR-Script from Stratagene GmbH, Heidelberg. The correctness of the sequence was checked by sequencing. It was transferred from the PCR-Script vector as XbaI fragment into the correspondingly cut vector which already contained, as described above, the DOXS sequence. The result was the construct pBinAR-DOXS-GGPPOR (Figure 15), whose fragments have the following significance:

Fragment A (529 bp) comprises the 35S promoter of cauliflower mosaic virus (nucleotides 6909 to 7437 of cauliflower mosaic virus). Fragment B comprises the transit peptide of the plastidic transketolase. Fragment E comprises the DOXS gene. Fragment D comprises the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription. Fragment F comprises the GGPPOR gene including the intrinsic plastid transit sequence.

Example 17

Production of constructs for transformation of plants with DOXS, GGPPOR and HPPD DNA sequences

To produce plants which are transgenic for DOXS, GGPPOR and HPPD, a binary vector containing all three gene sequences was manufactured (Figure 16). The GGPPOR gene was provided with the intrinsic plastidic localization sequence (as described in Example 15). The pBinAR-Hyg vector used confers on plants resistance to the antibiotic hygromycin and is thus suitable for superinfection of plants with kanamycin resistance.

To clone HPPD into vectors which additionally contain another cDNA, oligonucleotides were derived for a PCR, and a BamHI restriction cleavage site was attached to them at the 5' end and 3' end. The oligonucleotide at the 5' end comprises the sequence 5'-GG*ATCCTCCAGCGGACAAGCCAAC*-3' (nucleotides 37 to 55 distant from ATG in the 5' direction; in italics), and the oligonucleotide at the 3' end comprises the sequence 5'-ATGGATCC*CGCGCCGCCTACAGGTTG*-3' (ending with base pair 1140 of the coding sequence, starting 8 base pairs 3' of the TAG stop codon; in italics). The PCR reaction was carried out with Tli polymerase from Promega GmbH, Mannheim in accordance with the manufacturer's information. 10 ng of the plasmid pBinAR-HPPD were employed as template. The PCR program was as follows:

5 cycles: 94°C 4 sec, 68°C 30 sec, 72°C 2 min
5 cycles: 94°C 4 sec, 64°C 30 sec, 72°C 2 min
25 cycles: 94°C 4 sec, 60°C 30 sec, 72°C 2 min

The fragment was purified using a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script from Stratagene GmbH, Heidelberg. The correctness of the sequence was checked by sequencing. It was cut out of the vector PCR-Script as BamHI fragment and ligated into a correspondingly cut pBinAR vector which additionally contains the transit peptide of transketolase for introducing the gene product into plastids. The result was the plasmid pBinAR-TP-p-HPPD.

For the cloning, the 35S promoter, the transketolase transit peptide, the p-HPPD gene and the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al. 1984) for termination of transcription was isolated from the plasmid pBinAR-TP-p-HPPD by PCR. A HindIII cleavage site was attached in each case to the oligonucleotides for the promoter and the terminator. The sequence of the oligonucleotide which anneals onto the 5' region of the promoter (in italics) is 5'-ATAAGCTT*CATGGAGTCAAA-GATTCAAATAGA*-3', and that of the oligonucleotide which anneals onto the termination sequence (in italics) is 5'-ATAAGCTT*GGAC-AATCAGTAAATTGAACGGAG*-3'. The resulting fragment was purified using a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script from Stratagene GmbH, Heidelberg. The correctness of the sequence was checked by sequencing. It was transferred as HindIII fragment from this PCR-Script vector into the correspondingly cut vector pBin19 (Bevan, 1984, Nucleic Acids Res. 12, 8711-8721).

The 35S promoter, the transketolase transit peptide, the DOXS gene and the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription was isolated from the plasmid pBinAR-TP-DOXS by PCR. An EcoRI cleavage site was attached in each case to the oligonucleotides for the promoter and the terminator sequence. The sequence of the oligonucleotide which anneals onto the promoter (in italics) is 5'-ATGAATTC*CATGGAGTCAAAGATTCAAATAGA*-3', and that of the oligonucleotide which anneals onto the terminator sequence (in italics) is 5'-ATGAATTC*GGACAATCAGTAAATTGAACGGAG*-3'. The fragment was purified using a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script from Stratagene GmbH, Heidelberg. The correctness of the sequence was checked by sequencing. It was transferred from the PCR-Script vector as EcoRI fragment into the correspondingly cut vector which already contained the HPPD sequence as described above.

The 35S promoter, the GGPPOR gene and the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription was isolated from the plasmid pBinARHyg-GGPPOR by PCR. An XbaI cleavage site was attached in each case to the oligonucleotides for the promoter and the terminator. The sequence of the oligonucleotide which anneals onto the promoter (in italics) is 5'-ATTCTAGA*CATGGAGTCAAA-GATTCAAATAGA*-3', and that of the oligonucleotide which anneals onto the terminator sequence (in italics) is 5'-ATTCTAGA*GGACAA-TCAGTAAATTGAACGGAG*-3'. The fragment was purified using a Gene-Clean kit (Dianova GmbH, Hilden) and cloned in accordance with the manufacturer's information into the vector PCR-Script from Stratagene GmbH, Heidelberg. The correctness of the sequence was checked by sequencing. It was transferred from the PCR-Script vector as XbaI fragment into the correspondingly cut vector which already contained the HPPD and DOXS sequences as described above. The result was the construct pBinAR-DOXS-GGPPOR-HPPD (Figure 16), whose fragments have the following significance:

Fragment A (529 bp) comprises the 35S promoter of cauliflower mosaic virus (nucleotides 6909 to 7437 of cauliflower mosaic virus). Fragment B comprises the transit peptide of the plastidic transketolase. Fragment C comprises the HPPD gene. Fragment D comprises the polyadenylation signal of gene 3 of the T DNA of the Ti plasmid pTIACH5 (Gielen et al., 1984) for termination of transcription. Fragment E comprises the DOXS gene. Fragment F

comprises the GGPPOR gene including the intrinsic plastid transit sequence.

Example 18

Increasing tocopherol biosynthesis in oilseed rape

The cDNA of DOXS (SEQ ID No. 3) and of GGPOR (SEQ ID No. 7) was provided with a CaMV35S promoter and overexpressed in rape using the 35S promoter. In parallel with this, the seed-specific promoter of the phaseolin gene was used in order specifically to increase the tocopherol content in the rapeseed. Oilseed rape plants transformed with the corresponding constructs were grown in a glasshouse. The α-tocopherol content of the whole plant and of the seeds of the plant was then determined. In all cases, the α-tocopherol concentration was increased by comparison with the untransformed plant.

Example 19

Increasing the tocopherol biosynthesis in oilseed rape

The cDNA of DOXS (SEQ ID No. 3), of HPPD (SEQ ID No. 5) and of GGPPOR (SEQ-ID No. 7) was provided with a CaMV35S promoter and overexpressed in rape using the 35S promoter. In parallel with this, the seed-specific promoter of the phaseolin gene was used in order specifically to increase the tocopherol content in the rapeseed. Oilseed rape plants transformed with the corresponding constructs were grown in a glasshouse. The α-tocopherol content of the whole plant and of the seeds of the plant was then determined. In all cases, the α-tocopherol concentration was increased by comparison with the untransformed plant.